JOURNAL OF BACTERIOLOGY, Mar. 1980, p. 1192-1198
0021-9193/80/03-1192/07$02.00/0

Vol. 141, No. 3

Stimulation of Deoxyribonucleic Acid Replication Fork Movement by Spermidine Analogs in Polyamine-Deficient *Escherichia coli*

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We examined the rate of deoxyribonucleic acid (DNA) replication fork movement in polyamine-deficient cells of *Escherichia coli* by two independent techniques. DNA autoradiography was used to directly visualize the length of DNA produced during a given time interval, and replication rates were calculated. The amount of DNA synthesized after blocking protein synthesis also allowed calculation of replication rates. We found that the DNA chain elongation rate in polyamine-deficient cells was about half that of putrescine- or spermidine-supplemented cells. We also found that spermidine homologs of increasing chain length, when present at equal intracellular concentrations, exhibited a decreasing ability to support growth and the rate of DNA replication fork movement. The kinetics of recovery of DNA synthesis from the polyamine-deficient state were also investigated. A new rate of DNA synthesis was reached about 20 min after addition of spermidine to polyamine-limited cells. The rise in the rate of DNA synthesis was preceded by a rise in the intracellular concentration of spermidine.

The organic polycations putrescine and spermidine are present in millimolar concentrations in wild-type cells of the bacterium *Escherichia coli* (32), and mutants of this organism which require added polyamines for optimal growth have been isolated (4, 15, 16, 19, 20, 22). The physiology of these mutants has been studied in an attempt to identify the site(s) of polyamine action. Experiments from this laboratory, using steady-state, polyamine-limited *E. coli*, have shown that these cells have defects in three classes of macromolecular synthesis. The chain elongation rates for protein (10, 18), mRNA (18), and DNA (6) are all reduced in proportion to the reduction in growth rate. This is an unusual response, in that cells normally respond to a decreased growth rate by reducing the frequency of initiation of new chains and not by altering the chain growth rate. This has led to suggestions that polyamines may serve as cofactors in these chain growth processes (6, 18). Although these in vivo effects have been noted, none of the three classes of macromolecular synthesis has been established as a primary site of polyamine action. Indeed, it is possible that affecting only one of these processes could influence the others, since DNA replication, transcription, and translation are physically and temporally linked in *E. coli*.

We previously concluded that polyamine-deficient cells show a twofold reduction in the rate of DNA replication fork movement (6). In this paper, we substantiate this conclusion through the use of DNA autoradiography. This technique is more direct than those we previously used. We have also examined homologs of spermidine for their ability to support the DNA chain elongation rate in vivo. These homologs are triamines of the structure $NH_2(CH_2)_3NH(CH_2)_xNH_2$, where x is an integer from 4 to 8 (spermidine has x equal to 4). They exhibit a definite specificity, in that with increasing chain length there is a loss of ability to replace spermidine in maintaining growth and the rate of DNA replication fork movement. The kinetics of DNA synthesis and the size of polyamine pools during recovery from the polyamine-deficient state have also been investigated.

MATERIALS AND METHODS

Strains and growth conditions. *E. coli* strain DK6 was constructed by transduction of a biosynthetic arginine decarboxylase mutation (*speA*) into an antibiotic-susceptible strain of *E. coli* B (20). DK6 is incapable of synthesizing ornithine (20) and is therefore blocked in both pathways of putrescine biosynthesis (22) when grown in the presence of arginine. DK6 Thy$^-$, a low-level thymine-requiring derivative of DK6, was isolated in two steps. A high-level thymine auxotroph (60 μg/ml) was obtained by trimethoprim selection (27). This was then used to isolate a low-level thymine-requiring strain (2 μg/ml) by selecting for growth in media containing thymine at a concentration of 2 μg/ml. All experiments done with thy-

mine-requiring bacteria were done with the low-level auxotroph.

Cells were cultured at 37°C with rotary shaking, and growth was monitored by measuring turbidity at 450 nm. The morpholinopropane sulfonic acid (MOPS)-buffered medium of Neidhardt et al. (23), containing 0.2% glucose and an amino acid mix (15), was used for growth. Thymine was supplied at 2 μg/ml for thymine-requiring strains. Polyamine starvation was accomplished by overnight growth in the absence of added polyamines as previously described (20). When present, polyamines were added to give the following final concentrations: putrescine, 100 μg/ml; spermidine, 0.6 or 50 μg/ml as indicated in the text; *N*-(3-aminopropyl)-1,5-diaminopentane (AP5), 0.6 μg/ml; *N*-(3-aminopropyl)-1,6-diaminohexane (AP6), *N*-(3-aminopropyl)-1,7-diaminoheptane (AP7), and *N*-(3-aminopropyl)-1,8-diaminooctane (AP8), 100 μg/ml. The synthesis of these compounds has been described (9). The indicated concentrations of the spermidine homologs and 0.6 μg of spermidine per ml all give rise to similar intracellular concentrations of the triamines (9).

Autoradiography of DNA. DNA autoradiography was carried out according to the method of Prescott and Kuempel (24), and the cellular DNA was labeled according to protocol B of Lane and Denhardt (12). Polyamine-starved and unstarved log-phase cells of DK6 Thy$^-$ (1.0 ml of 5×10^7 cells/ml) were filtered and resuspended in 1.0 ml of prewarmed medium containing [^{3}H]thymine (4.1 μg/ml, 7.7 Ci/mmol). Further growth was allowed for 35 min, at which time the cells were added to a prewarmed tube containing [^{3}H]thymidine (500 ng/ml, 90 Ci/mmol). Further incubation was for 3.0 min in the case of unstarved cells and 6.0 min in the case of polyamine-starved cells. The high-specific-activity [^{3}H]thymidine pulse label was terminated by addition of an equal volume of ice-cold buffer containing 50 mM Tris-hydrochloride (pH 8.1), 2 mM EDTA, and 10 mM NaN_3. The cells were centrifuged at 4°C and washed twice with cold Tris/EDTA/NaN_3, and spheroplasts were prepared (11). The spheroplasts were lysed on a subbed microscope slide (2) by adding a drop of 10% sodium dodecyl sulfate. After 10 min the drop was spread with the edge of a cover glass. The slide was then dried, washed three times with cold 5% trichloroacetic acid and once with cold 95% ethanol, and allowed to dry. The dried slide was then coated with Kodak NTB2 emulsion and allowed to expose for about 6 months. The developed slides were scanned at ×400 magnification, using bright-field illumination. Allowable tracks (see below) were traced with the aid of a camera lucida, and the track lengths were measured by comparison with a stage micrometer. Those grain tracks which were recorded consisted only of one high-density region connected to a low-density region or only of two high-density regions flanking a low-density region. In addition, the recorded tracks exhibited no aggregated regions. Recording of tracks was done with coded slides so that track selection and measurement was done without knowledge of the source of the samples.

Increment in DNA synthesis after blocking protein synthesis (Δ*G*). The cellular DNA of DK6 was labeled by growing the cells in the presence of [^{3}H]thymine (2 μg/ml, 5.75 mCi/mmol) and deoxyguanosine (500 μg/ml). Deoxyguanosine in high concentration allows these Thy$^+$ cells to incorporate the labeled thymine (35).

After approximately three to four generations of growth in radioactive medium, protein synthesis was inhibited by addition of puromycin or chloramphenicol to 200 μg/ml (from a stock solution of 2 mg/ml in MOPS buffer). Three 1-ml samples were immediately taken into 1 ml of ice-cold 10% trichloroacetic acid for zero time points. At measured intervals thereafter, additional 1-ml samples were collected until DNA synthesis ceased.

Acid-precipitable material was collected by filtering through Schleicher & Schuell glass fiber filters (no. 25). The filters were then washed with approximately 100 ml of cold 5% trichloroacetic acid containing 5 mM $Na_4P_2O_7$ and washed once with cold 95% ethanol. The filters were subsequently dried and counted in a liquid scintillation cocktail containing 5.1 g of PPO (2,5-diphenyloxazole) per liter of toluene.

Δ*G* was calculated as the ratio of the plateau value of [^{3}H]thymine incorporated after cessation of DNA replication to that value obtained at time zero, i.e., the time of puromycin addition. The replication rate was calculated as described in the text.

Spermidine shift-up experiments. A polyamine-starved overnight culture of DK6 was used to inoculate media lacking polyamines and containing $^{32}P_i$ (1.32 mM, 1.52 mCi/mmol). This culture was then divided into three parts: (i) cells for an unstarved control (spermidine added to 50 μg/ml at the time the culture was divided), (ii) cells for a starved control, and (iii) cells for the culture to be shifted. Growth was allowed to continue for four to five generations (to 5×10^7 to 10×10^7 cells/ml) in all three cultures, and at time zero the starved cells in the third culture were shifted by adding spermidine to a final concentration of 50 μg/ml. The rate of DNA synthesis during the transition was monitored by removing 1.0 ml of cells to a tube (at 37°C) containing [^{3}H]adenosine (20 μCi, 12.3 Ci/mmol). The pulse was terminated after 1.0 min by addition of an equal volume of ice-cold 10% trichloroacetic acid. Since [^{3}H]adenosine and $^{32}P_i$ will also label RNA, samples were centrifuged and the pellet was resuspended in 1.0 ml of 0.6 M NaOH. The samples were then placed at 37°C overnight to hydrolyze RNA. The samples were then trichloroacetic acid precipitated by addition of 1.0 ml of 20% trichloroacetic acid containing 0.6 M HCl, filtered, and counted as described above.

Polyamine pools during the spermidine shift-up experiment were measured by pouring 30 ml of culture (3×10^9 cells) into 5 g of ice and 3 ml of 0.10 M NaN_3. The presence of NaN_3 prevents the acetylation which is usually observed when cells are harvested at low temperature (29). In a control experiment (data not shown) performed on steady-state cultures, this stopping technique yielded the same pool values as cells that were harvested at room temperature, and there was no evidence for the formation of the acetyl spermidine isomers. Cells harvested on ice and NaN_3 were then trichloroacetic acid extracted and analyzed for

polyamines as previously described (17, 21). The described analytical procedure has been modified in that a different buffer and resin system (31), which allows for increased resolution of diamines, was used.

A control experiment was run in which a starved culture was first poured onto ice and NaN_3 and then spermidine was added to 50 μg/ml (data not shown). These cells contained 10.7 nmol of spermidine per mg of protein more than the starved cultures. This difference was attributed to electrostatic binding to the cell surface rather than true uptake (30) and was subtracted from the pool values of shifted cultures.

Protein determinations were made according to the method of Lowry et al. (13), using bovine serum albumin as a standard.

RESULTS

DNA autoradiography. Unstarved and polyamine-starved cells were continuously labeled with low-specific-activity [^{3}H]thymine and then pulse-labeled with high-specific-activity [^{3}H]-thymidine (see Methods and Materials). The starved cells were pulse-labeled for 6 min and the unstarved cells for 3 min in order to obtain approximately equal track length distributions. Figure 1 shows examples of grain tracks which were recorded for length measurements. Most of the tracks consisted of a high-density region joined to a low-density region, but some (2 out of 95 for putrescine-supplemented cells and 6 out of 88 for polyamine-starved cells) consisted of two-high density regions flanking a low-density region. We presume that this pattern represents bidirectional replication from an intitiation event which occurred during labeling with low-specific-activity thymine (24).

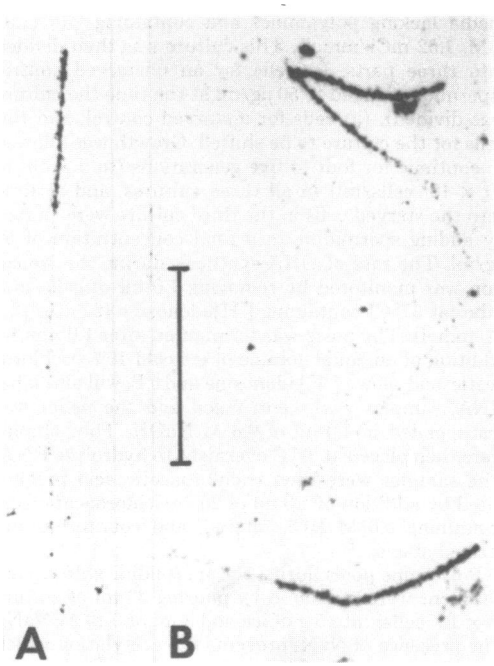

FIG. 1. *Grain tracks produced by autoradiography of cellular DNA labeled with ^{3}H as described in Materials and Methods. The above tracks illustrate the types of grain tracks which were included in the data of Fig. 2. (A) A high-density region joined to a low density region; (B) two high-density regions flanking a low-density region. The bar indicates a length of 25 μm.*

Rates were calculated by dividing the track length by the pulse time, and these rates were used to construct the histograms shown in Fig. 2. The average rate for putrescine-supplemented cells was 11.5 ± 0.6 μm/min (mean ± standard error of the mean), and the value for polyamine-deficient cells was 5.9 ± 0.4 μm/min. In an independent experiment (data not shown), starved cells were pulse-labeled for 3 min, rather than 6 min, and a replication rate of 4.7 ± 0.8 μm/min (sample size of 50 tracks) was calculated from the measurements. Thus, as was concluded previously from other, less direct measurements, the average rate of DNA chain elongation for cells supplemented with putrescine was twice that of polyamine-deficient cells.

ΔG. The amount of DNA synthesized after blocking protein synthesis (ΔG) is a function of the number of rounds (n) of DNA replication in progress (14); the more rounds in progress, the larger the increment will be [$\Delta G = 2^n \cdot n \cdot \ln 2/2^n - 1$)] (28). Knowing n and the generation time, τ, of the culture allows one to calculate C, the replication time [$n = C/\tau$].

Figure 3 shows the residual synthesis of DNA after blocking protein synthesis with puromycin. The ΔG values from this experiment translate into C values of 48 min for unstarved cells and

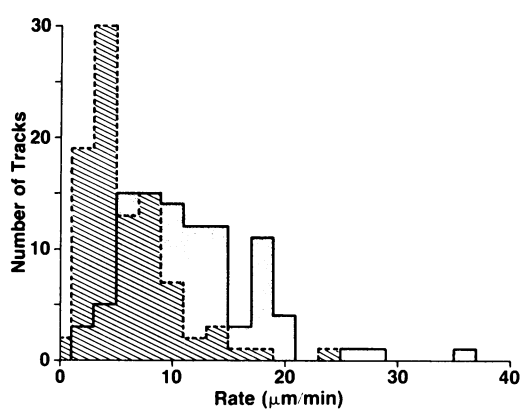

FIG. 2. *DNA replication rates in polyamine-starved (- - -) and putrescine-supplemented (—) cells. The rates were calculated by dividing the high-density track length by the duration of the high-specific-activity [^{3}H]thymidine pulse.*

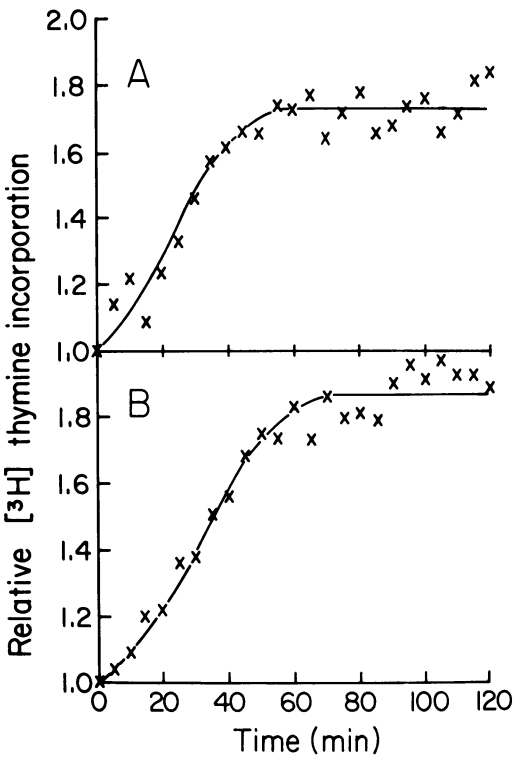

FIG. 3. *Increment in DNA synthesis after blocking protein synthesis (ΔG). At time zero, puromycin was added to cells growing in medium containing [^{3}H]-thymine (see Materials and Methods). The data were plotted as the ratio of the [^{3}H]thymine incorporated at the particular time after inhibitor addition to the [^{3}H]thymine incorporated at time zero. ΔG is the final plateau value of this ratio and is used to calculate C as described in the text. (A) Spermidine-supplemented cells (cpm at t_0 = 12,000); (B) polyamine-starved cells (cpm at t_0 = 6,640).*

92 min for starved cells. Similar results were obtained (results not shown) after using chloramphenicol in place of puromycin and strain DK6 Thy$^-$ in place of DK6 (which obviates the need for deoxyguanosine at 500 μg/ml). These *C* values converted to replication rates of 14.5 μm/min for spermidine-supplemented cells and 7.6 μm/min for polyamine-starved cells (Table 1). Putrescine-supplemented cells had a replication rate of 12.6 μm/min (data not shown). These values are in reasonable agreement with those obtained with DNA autoradiography.

DNA replication rates in the presence of spermidine homologs. The Δ*G* technique was used to measure the ability of homologs of spermidine to support the rate of DNA replication in vivo (Table 1). The experiments were performed at concentrations of the triamines in the culture media which gave roughly equivalent intracellular pools (Materials and Methods). AP5 supported replication at a rate intermediate between that of starved and unstarved cells. AP6-supplemented cells had an elongation rate similar to that of starved cells, and the rates with AP7 and AP8 were slightly slower than that seen with starved cells. In general, the DNA chain growth rate declined as the growth rate became slower and the number of rounds of replication in progress remained roughly constant.

Kinetics of recovery of DNA synthesis from polyamine starvation. Spermidine (50 μg/ml) was added to cultures of polyamine-starved DK6, and the rate of DNA synthesis was measured as a function of recovery time (Fig. 4). The cells were continuously labeled with $^{32}P_i$ and then were pulse-labeled with [^{3}H]adenosine to monitor DNA synthesis during the shift. [^{3}H]adenosine, rather than [^{3}H]thymidine, was used to label the DNA because the ATP pools are the same in starved and unstarved cells, whereas the TTP pools are not (D. R. Morris and C. M. Jorstad, unpublished data). There was a definite increase in [^{3}H]adenosine incorporation by 7 min after addition of spermidine,

TABLE 1. *Influence of spermidine homologs on DNA replication rates*[a]

Polyamine	μ[b]	Δ*G*	*C* (min)	Replication rate (μm/min)[c]
No addition	1.33	1.87	92	7.6
Spermidine[d]	2.22	1.73	48	14.5
AP5[e]	1.79	1.96	74	9.4
AP6	1.45	1.98	93	7.6
AP7	1.28	2.18	124	5.7
AP8	0.96	1.84	124	5.7

[a] Determination of Δ*G* and calculation of *C* were performed as described in the text. The values for the spermidine homologs represent the mean of two independent determinations done with puromycin. The values for polyamine-starved and spermidine-supplemented cells are the results of single experiments with puromycin, but they are in close agreement with extensive data gathered using chloramphenicol (see text). Values for a given set of experimental conditions differ from the mean by not more than 10%.

[b] Growth rate, expressed as mass doubling per hour.

[c] Values of 4.1 × 10^6 base pairs (1) and 0.34 nm per base pair give a length of 1,390 μm for the *E. coli* genome. The rate calculation is made by assuming that each replication fork travels 695 μm during a round of replication.

[d] Spermidine was added to the culture medium at 0.6 μg/ml to give an intracellular pool size equivalent to that of the other triamines (see Materials and Methods).

[e] Nomenclature of the homologs is as previously described (9).

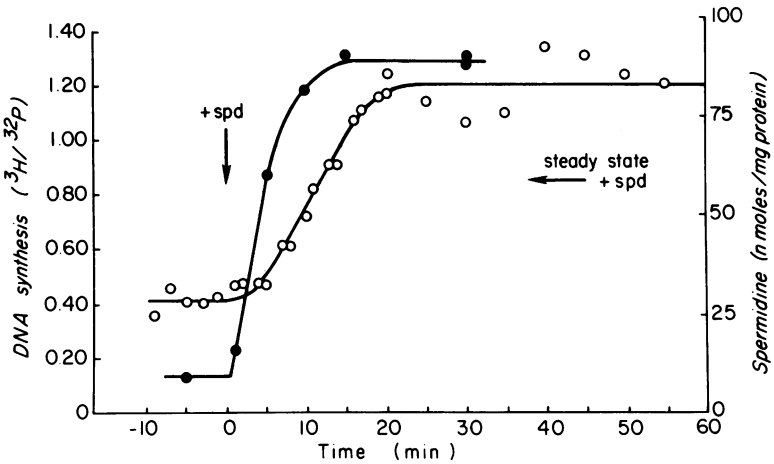

FIG. 4. *Rate of DNA synthesis and intracellular spermidine pools after addition of spermidine to a starved culture. The incorporation of [^{3}H]adenosine into DNA* (○) *was measured after a 1.0-min pulse label. $^{32}P_i$ was present as a continuous label for four generations before the shift, and the ^{32}P incorporated was therefore proportional to the amount of DNA in the sample. The incorporation in a steady-state culture grown in the presence of spermidine (spd) is indicated by the arrow. Spermidine pools* (●) *were measured as described in Materials and Methods.*

and it may have begun sooner. The transition to the new rate of DNA synthesis was complete in about 20 min. The $^3H/^{32}P$ ratio for a steady-state unstarved culture is indicated by the arrow in Fig. 4. This value was about twice (0.87 versus 0.41) that of starved cells, but lower than the maximum rate achieved by the shifted culture. The difference in steady-state values was expected since the unstarved cells accumulated DNA at about twice the rate of starved cells (6). The reason for the overshoot routinely seen in the shifted cultures is presently unexplained.

Spermidine uptake was studied after supplying the amine to starved cells (Fig. 4). Pool levels almost doubled by 30 s after spermidine addition and were 70% of maximum by 5 min. The rise in intracellular spermidine level clearly preceded the increase in the rate of DNA synthesis.

DISCUSSION

Three independent techniques, DNA autoradiography (this paper), ΔG (this paper and reference 6), and segregation of labeled chromosomes (6), have led to the conclusion that the rate of DNA replication fork movement is slowed by a factor of about two in polyamine-starved cells. At present there is no compelling evidence for or against a direct interaction of spermidine with the DNA replication machinery in vivo. It is certainly possible that the observed effect on DNA replication fork movement is removed from, and secondary to, the actual site(s) of spermidine action.

We studied the specificity of the spermidine requirement in vivo by using the spermidine homologs described by Jorstad et al. (9). A definite specificity was noted; as the length of the carbon chain increased, there was a decreased ability to support DNA replication. Thus, there appears to be a requirement for a particular arrangement of the amino groups, and it is not the case that any triamine will serve the role(s) of spermidine. A similar specificity was also seen when these spermidine homologs were tested for their ability to support mRNA and protein chain elongation rates (9).

DNA synthesis in starved cells responded rapidly to the addition of polyamines (Fig. 4), reaching a new level about 20 min after spermidine addition. This is in marked contrast to an earlier report where DNA synthesis remained at the starved rate for 2 h after putrescine addition (35). The significance of this difference is hard to interpret for several reasons. Different strains were used which have grossly different growth properties; putrescine-starved DK6 doubles every 50 to 60 min, whereas strain MA135, used by the previous workers, had a doubling time of 150 to 300 min when putrescine starved. These growth differences appear to be unrelated to the state of polyamine deficiency since both strains have virtually no putrescine and greatly reduced spermidine (20, 35). The strains have other physiological differences in that MA135 is filamentous upon polyamine starvation, whereas DK6 is not. In addition, the previous workers shifted by adding putrescine, whereas we used spermi-

dine. Part of the lag observed by these workers might have been due to the time required for putrescine to be converted to spermidine, although the absence of pool data in their paper makes this impossible to evaluate.

Spermidine is known to stimulate several in vitro reactions which may be related to DNA synthesis in vivo. The conversion of single-stranded phage DNA to its replicative form (5, 25, 26, 34), the reannealing of DNA by *E. coli* helix destabilizing protein under physiological conditions (3), and the DNA gyrase reaction (7, 8) are all enhanced by spermidine in vitro. The tandem use, both in vivo and in vitro, of the spermidine homologs may shed light on the issue of the in vivo relevance of the spermidine stimulation of these in vitro systems.

ACKNOWLEDGMENTS

This work was supported in part by Public Health Service grant GM 13957 from the National Institute of Health (GM 13957). L. G. was supported by Public Health Service training grants GM 00052 and GM 07270 from the National Institutes of Health.

We thank Caroline M. Jorstad for performing the polyamine analyses.

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